immunostaining of whole embryos (12/101 antibody was obtained from the Developmental Studies Hybridoma Bank, NICHD), and RT-PCR analysis of RNA extracted from cultured animal caps was done as described[25] except that 14-μm sections were cut and PCR cycles were as follows: 95 °C for 5 min followed by a variable number of cycles (determined empirically for each primer pair) at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Control reactions in which reverse transcriptase was omitted were run in parallel for all samples. The sequences of *EF1-α*, *N-CAM*[26]; α-actin[27]; *OtxA*[28]; *XAG1* (ref. 29); *Xbra*[30] primers have been reported previously.

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Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling

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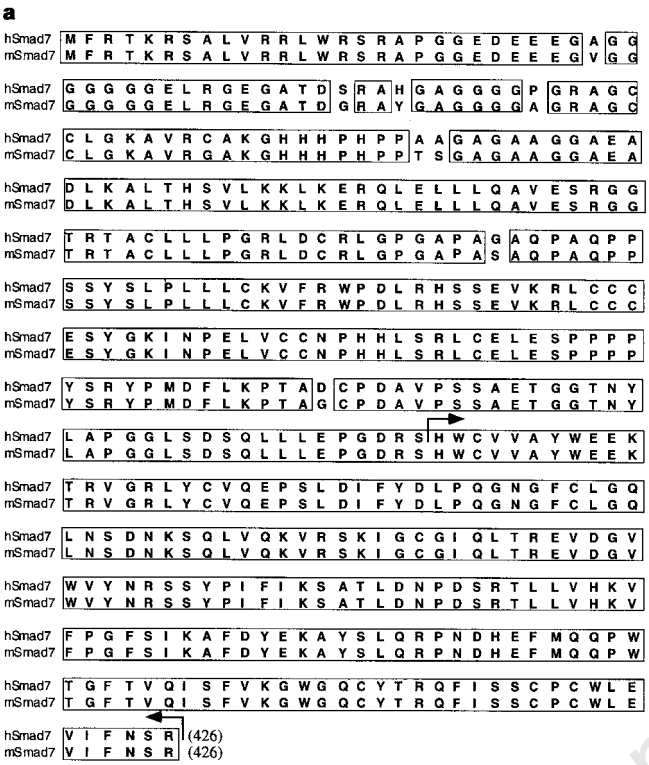
TGF-β signals from the membrane to the nucleus through serine/threonine kinase receptors and their downstream effectors, termed SMAD proteins[1]. The activated TGF-β receptor induces phosphorylation of two such proteins, Smad2 and Smad3 (refs 2–6), which form hetero-oligomeric complex(es) with Smad4/DPC4 (refs 5–10) that translocate to the nucleus[2,4,5,7], where they then regulate transcriptional responses[11,12]. However, the mechanisms by which the intracellular signals of TGF-β are switched off are unclear. Here we report the identification of Smad7, which is related to Smad6 (ref. 13). Transfection of Smad7 blocks responses mediated by TGF-β in mammalian cells, and injection of Smad7 RNA into *Xenopus* embryos blocks activin/TGF-β signalling. Smad7 associates stably with the TGF-β receptor complex, but is not phosphorylated upon TGF-β stimulation. TGFβ-mediated phosphorylation of Smad2 and Smad3 is inhibited by Smad7, indicating that the antagonistic effect of Smad7 is exerted at this important regulatory step. TGF-β rapidly induces expression of Smad7 mRNA, suggesting that Smad7 may participate in a negative feedback loop to control TGF-β responses.

Smad7 was identified as an expressed sequence tag (EST) related to known SMAD proteins. Sequence analysis of isolated mouse and human cDNAs predict that mouse Smad7 and human Smad7 have 426 amino-acid residues with 98% identity (Fig. 1a). Smad7 is most related to Smad6 (ref. 13), with 36% and 56% sequence identities in the amino-terminal domain and the carboxy-terminal Mad homology (MH)2 domain, respectively. The Smad7 amino-terminal domain shows only weak similarity to the MH1 domains found in Smad1 to Smad5. RNA blot analysis with a Smad7 probe revealed one main transcript of approximately 4.4 kilobases (Fig. 1b). Among the tissues analysed, the highest expression of *Smad7* was found in the lung.

In order to investigate whether Smad7 modulates the responsiveness to TGF-β, we transfected the TGFβ-inducible p3TPLux luciferase reporter construct, which contains the PAI-1 promoter, into Mv1Lu mink epithelial cells in the absence or presence of Smad7 cDNA. Smad7 was found to inhibit TGFβ1-induced luciferase activity (Fig. 2a). Moreover, the induction of p3TPLux response by constitutively active variants of the TGF-β receptor TβR-I and a structurally related type IB receptor for activin (ActR-IB), when transfected in R-mutant cells, was also inhibited by co-transfection with Smad7 (Fig. 2b, and data not shown). Thus our results indicate that Smad7 is a potent negative regulator of p3TPLux response induced by both TβR-I and ActR-IB. In addition, Smad7, but not



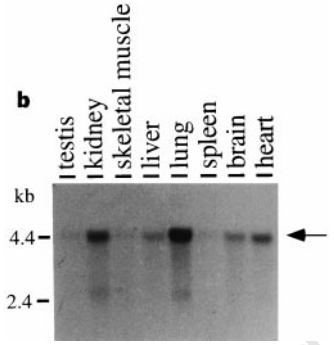
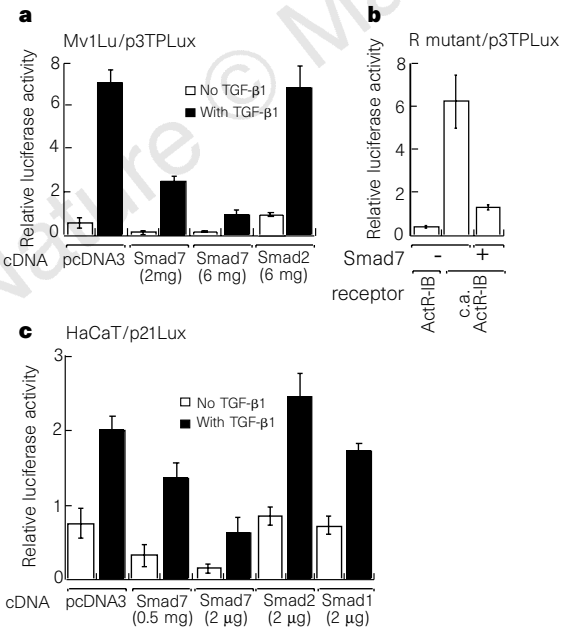


Figure 1 Amino-acid sequence of Smad7 and expression of Smad7 mRNA. **a**, Predicted amino-acid sequence of mouse (m) and human (h) Smad7. The borders of the Mad-homology (MH)2 domain are indicated by arrows. Genbank accession numbers for mouse and human Smad7 are AF015260 and AF015261, respectively. **b**, Expression of Smad7 mRNA in various mouse tissues. A blot with mRNAs from mouse tissues was hybridized with an N-terminal domain Smad7 probe.



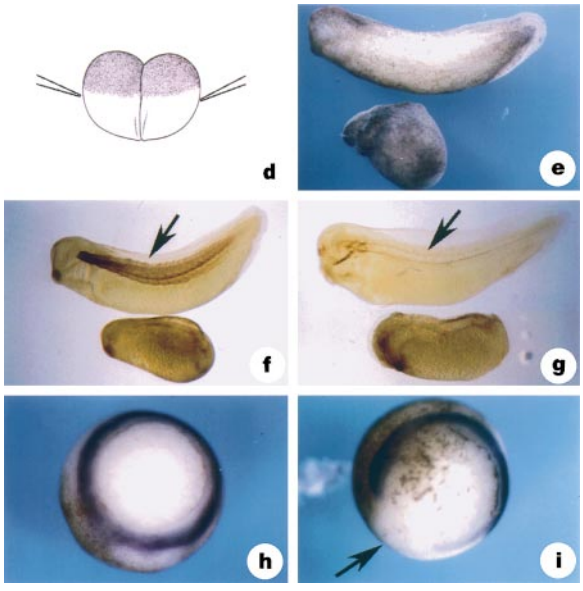


Figure 2 Effects of mouse Smad7 transfection on responses induced by TGF-β family members, and effects of RNA injection in embryos. **a**, Transfection of Smad7, but not Smad2, in Mv1Lu cells blocks the TGFβ-induced p3TPLux response. **b**, Transfection of Smad7 in R mutant cells inhibits p3TPLux response induced by constitutively active (c.a.) ActR-IB. **c**, Transfection of Smad7, but not Smad 2 or Smad1, in HaCaT cells antagonizes the TGFβ-induced p21 Lux response. **d**–**g**, Injection of 500 pg synthetic RNA encoding either Smad7 or myc-epitope tag (MT)[29] into each blastomere of two-cell embryos, as shown schematically (**d**); embryos were cultured to the tailbud stage. Embryos injected with MT RNA developed normally (top embryo in each panel), whereas embryos made to misexpress Smad7 (bottom embryo in each panel) failed to form head or tail structures (**e**) and showed a complete or partial loss of muscle (**f**) and notochord (**g**). Arrows indicate immunoreactive muscle (**f**) and notochord (**g**) in MT-injected embryos. **h**, **i**, Injection of 500 pg synthetic RNA encoding MT (**h**) or Smad7 (**i**) into one blastomere of two-cell embryos near the equator. Expression of *brachyury* was analysed by whole-mount *in situ* hybridization. MT-injected embryos show the typical ring of *brachyury* expression (**h**), but *brachyury* transcripts are not detected on one side of Smad7-injected embryos (**i**, arrow).

Smad1 or Smad2, was found to antagonize the TGFβ-induced transcriptional response with a luciferase reporter construct containing the p21 CDK inhibitor promoter (p21Lux)[13] in human keratinocytes (HaCaT) (Fig. 2c). Smad7 and N-terminally Flag-tagged Smad7 (F-Smad7) gave essentially the same antagonistic results on TGF-β signalling, suggesting that N-terminal tagging does not interfere with Smad7 function. Thus Smad7 inhibits TGFβ-induced pathways leading to extracellular matrix production as well as growth inhibition.

To determine whether Smad7 can inhibit TGF-β/activin-like signals *in vivo*, we analysed patterning defects caused by overexpression of Smad7 in *Xenopus* embryos. When the endogenous activin signalling pathway is inactivated in early *Xenopus* embryos, by introduction of dominant-negative forms of either an activin receptor[14,15] or of Smad4 (ref. 6), mesoderm fails to form. Similarly, microinjection of RNA encoding Smad7 into both blastomeres of two-cell embryos inhibited mesoderm formation. Specifically, head and tail structures were absent or severely deficient in 80% of injected embryos ($n = 168$; Fig. 2e), as were mesodermal derivatives such as muscle ($n = 60$; Fig. 2f) and notochord ($n = 48$; Fig. 2g). To test further the ability of Smad7 to block mesoderm formation *in vivo*, we analysed embryos for expression of *brachyury*, a gene that is expressed throughout the presumptive mesoderm during gastrulation[16] (Fig. 2h). Injection of Smad7 RNA into the equatorial region of one blastomere of two-cell embryos prevented *brachyury* expression on one side of the embryo (Fig. 2i).

Two members of the TGF-β family, activin and Vg1, are candidates for being endogenous mesoderm-inducing molecules[17]. We used a *Xenopus* animal-cap assay[17] to test directly the ability of Smad7 to block signalling downstream of these ligands. Both activin and Vg1 induced expression of *brachyury* in ectodermal explants (animal caps), but coexpression of Smad7 inhibited brachyury induction by 60% (data not shown), demonstrating that Smad7 can block activin- and Vg1-mediated mesoderm induction.

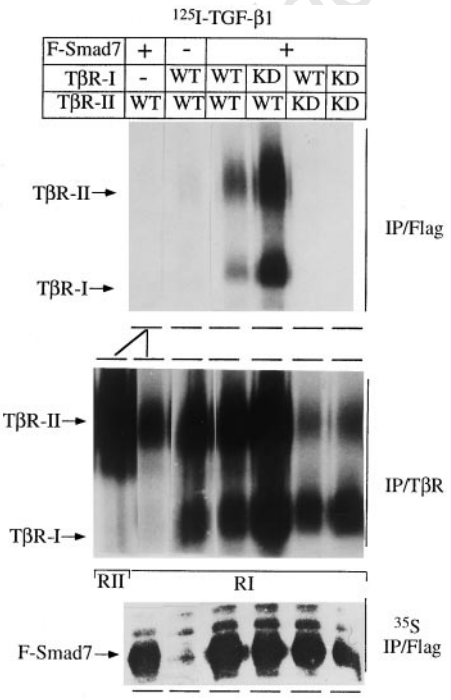


Figure 3 Association of Smad7 with the TGF-β receptor complex. COS cells were transfected with F-Smad7 in combinations of wild-type (WT) or kinase-deficient (KD) mutants of TβR-II and TβR-I. The receptors were covalently affinity labelled with ^{125}I-TGFβ1. Immunoprecipitates were analysed by SDS–PAGE and FujiX Bio-Imager. Expression of receptors and F-Smad7 after transfection was determined by immunoprecipitation (IP) with specific antisera on lysates from cells affinity-labelled with ^{125}I-TGF-β1 or [^{35}S]methionine/cysteine-labelled transfected cells, respectively.

To investigate the mechanism by which Smad7 exerts its negative role in signalling by TGF-β family members, we tested whether Smad7, like Smad2 and Smad3 (refs 3–5), can associate with the TGF-β receptor complex. COS cells transfected with F-Smad7 in combinations with TβR-II (wild-type or kinase-deficient mutant) and TβR-I (wild-type or kinase-deficient mutant) were affinity-labelled with ^{125}I-TGFβ1, and cell lysates were immunoprecipitated with Flag antiserum. We found that Smad7 interacted very efficiently with the TGF-β receptor complex (Fig. 3). Smad7 interacted with wild-type TβR-I as well as kinase-inactive TβR-I in complex with wild-type TβR-II. In contrast, Smad2 and Smad3 interact stably only with complex of kinase-deficient TβR-I and wild-type TβR-II (refs 4, 5). No interaction was observed between Smad7 and a heteromeric complex of kinase-deficient TβR-II and TβR-I, or with TβR-II alone (Fig. 3). Thus transphosphorylation of TβR-I by TβR-II kinase is required for the association of Smad7 with TβR-I.

We investigated whether Smad7 is phosphorylated upon association with TGF-β receptors. COS cells were transfected with Smad7, or with Smad2 for comparison, in the absence or presence of receptors, labelled with [^{32}P]orthophosphate, and treated with TGF-β1. As expected, Smad2 phosphorylation was increased upon coexpression with receptors, and addition of TGF-β1 led to a further increase[2,4–6]. However, we observed no (or only very weak) phosphorylation of Smad7 in cells transfected with constitutively active forms of TβR-I and ActR-IB, or in cells transfected with wild type TβR-I and TβR-II after stimulation with ligand (Fig. 4a). Thus, despite its direct association with TβR-I, Smad7 is not a substrate for the TGFβR-I kinase. Notably, Smad7 lacks the conserved SS(M/V)S motif in its carboxy tail (Fig. 1a), which in the case of Smad2 (ref. 4) and Smad1 (ref. 10) are phosphorylated by type-I receptor kinase.

Smad7 might exert its negative role in TGF-β signalling by interfering with activation of other SMAD proteins. We therefore examined whether Smad7 affects the phosphorylation of Smad2 and Smad3 by using [^{32}P]orthophosphate-labelled COS cells transfected with TGF-β receptors and SMAD proteins. Smad7 inhibited the TGFβ1-induced phosphorylation of Smad2 (Fig. 4b, c) and Smad3 (Fig. 4d, e). A Smad7 level twice as high as that of Smad3 was sufficient to cause a significant decrease in TGFβ-induced receptor-mediated phosphorylation of Smad3. As previously reported[4,5], we found that Smad2 and Smad3 become phosphorylated when overexpressed in COS cells, which for Smad2 does not occur at the SSMS motif in the C-terminal tail[4]. Smad7 appears not to block this receptor-independent phosphorylation. Because activation of Smad2 and Smad3 is essential for optimal TGF-β signalling[3–6,10], the inhibition of their phosphorylation provides a mechanistic explanation for the antagonistic effect of Smad7. The association of Smad7 with TβR-I suggests that it may compete with Smad2 and Smad3 for receptor binding. In accordance with this notion, co-transfection of Smad2 or Smad3 with Smad7 reduced the antagonistic effect of Smad7 in a TGFβ1-induced p3TPLux assay (data not shown). It has recently been reported that Smad7 inhibits TGF-β signalling responses[18], which is in agreement with our findings. We also found that Smad7 not only inhibits TGF-β and activin signalling, but also blocks signalling by bone morphogenetic proteins (BMPs). Smad7 associated with BMPR-Is and inhibited phosphorylation of Smad1 in cultured mammalian cells (A.N., unpublished results). In addition, injection of Smad7 RNA into ventral cells of *Xenopus* embryos phenocopies the effect of blocking the BMP signalling pathway, and led to formation of an incomplete secondary dorsal axis (J.L.C., unpublished results).

Regulation of Smad7 expression by signalling molecules may provide a possible means of effectively modulating TGF-β responses. We therefore investigated whether the expression of Smad7, and for comparison Smad2, Smad3 and Smad4, were

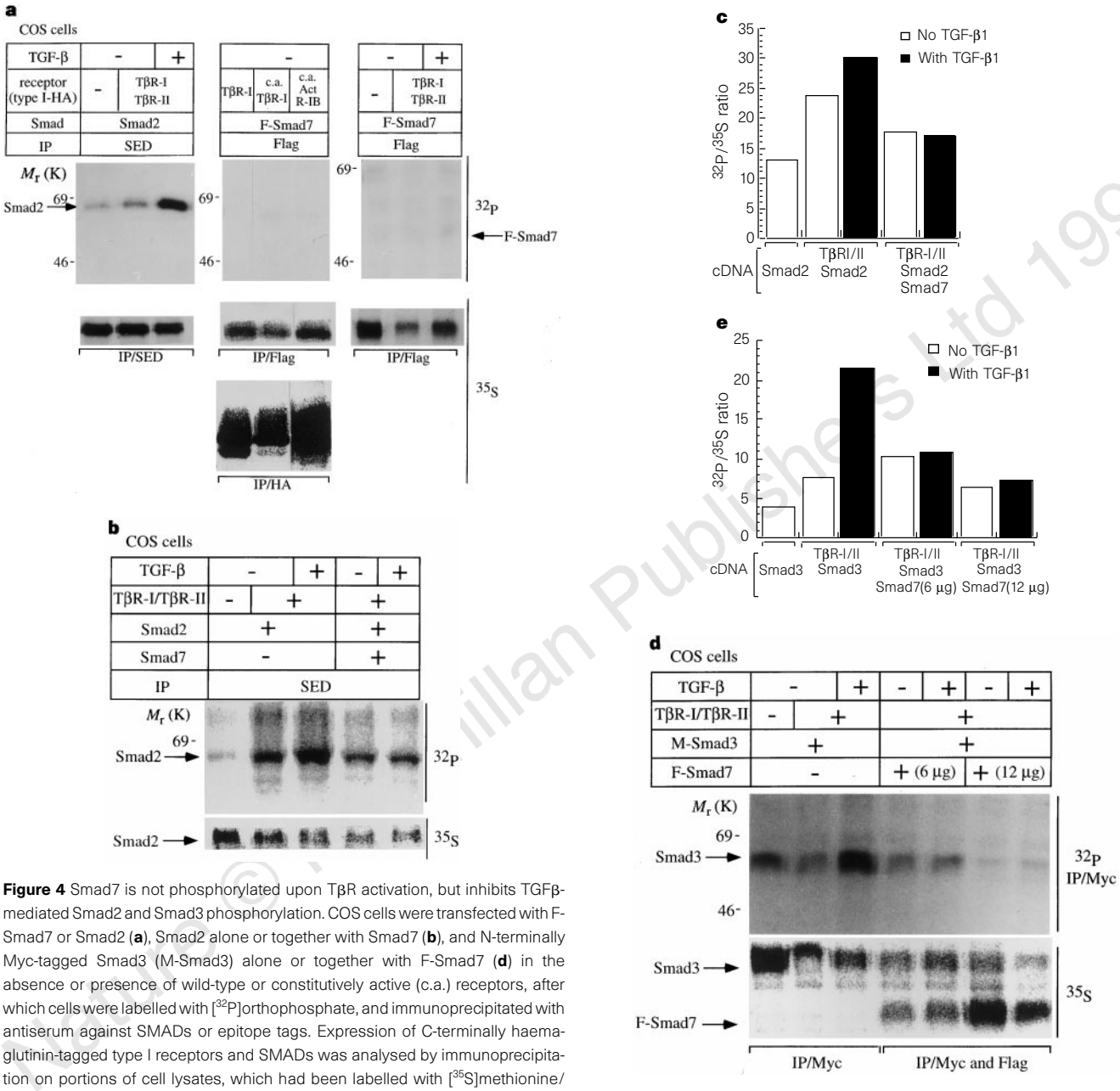


Figure 4 Smad7 is not phosphorylated upon TβR activation, but inhibits TGFβ-mediated Smad2 and Smad3 phosphorylation. COS cells were transfected with F-Smad7 or Smad2 (**a**), Smad2 alone or together with Smad7 (**b**), and N-terminally Myc-tagged Smad3 (M-Smad3) alone or together with F-Smad7 (**d**) in the absence or presence of wild-type or constitutively active (c.a.) receptors, after which cells were labelled with [^{32}P]orthophosphate, and immunoprecipitated with antiserum against SMADs or epitope tags. Expression of C-terminally haemaglutinin-tagged type I receptors and SMADs was analysed by immunoprecipitation on portions of cell lysates, which had been labelled with [^{35}S]methionine/cysteine. The ^{32}P or ^{35}S radioactivity associated with Smad2 in **b** or Smad3 in **d** was quantified by using a FujiX Bio-Imager, and the ^{32}P/^{35}S ratio was calculated (**c** and **e**, respectively). Representative results of three independent experiments are shown.

regulated by TGF-β1. Northern blot analysis of SMADs on RNA from TGFβ1-stimulated Mv1Lu cells, SW1736 human anaplastic thyroid carcinoma cells, and HaCat cells, revealed that Smad7 mRNA, but not Smad2, Smad3 or Smad4 mRNA, was rapidly induced in response to TGF-β1 stimulation (Fig. 5a, b). In Mv1Lu cells, Smad7 mRNA was induced fourfold more after 30 min of TGF-β stimulation. Induction of Smad7 mRNA by TGF-β1 was observed in the presence of cycloheximide (CHX), indicating that *de novo* protein synthesis is not required for this response. Smad7 mRNA was actually superinduced in the presence of TGF-β1 and CHX (Fig. 5b), probably as a result of an increase in mRNA stability or loss of transcriptional repressors by CHX. Treatment with CHX alone only slightly increased the basal Smad7 mRNA level (data not shown). In contrast, the phorbol ester TPA (12-*O*-tetradecamoylphorbol-13-acetate), which like TGF-β1 stimulates PAI-1 expression and inhibits the growth of Mv1Lu cells, did not induce Smad7 expression (Fig. 5c). Taken together, these data indicate that *Smad7* is an immediate-early response gene for TGF-β1, and may act in a negative feedback loop to regulate the intensity or duration of the TGF-β signal.

TGF-β-like signalling responses have been shown to be regulated at both the ligand level[19,20] and the receptor level[21–23]. Our results indicate that another important level of modulation of TGF-β signals may be exerted through intracellular agonistic and antagonistic SMADs. Loss of expression or overexpression of Smad7 may contribute to diseases in which TGF-β has been implicated. □

Methods

Isolation of mSmad7 and hSmad7 cDNA and northern blot analysis. cDNA encoding the complete mSmad7 was made by fusing mouse EST cDNA (AA061644) with a partial cDNA isolated from a mouse placenta library. The cDNA for hSmad7 was isolated by screening a human brain cDNA library.

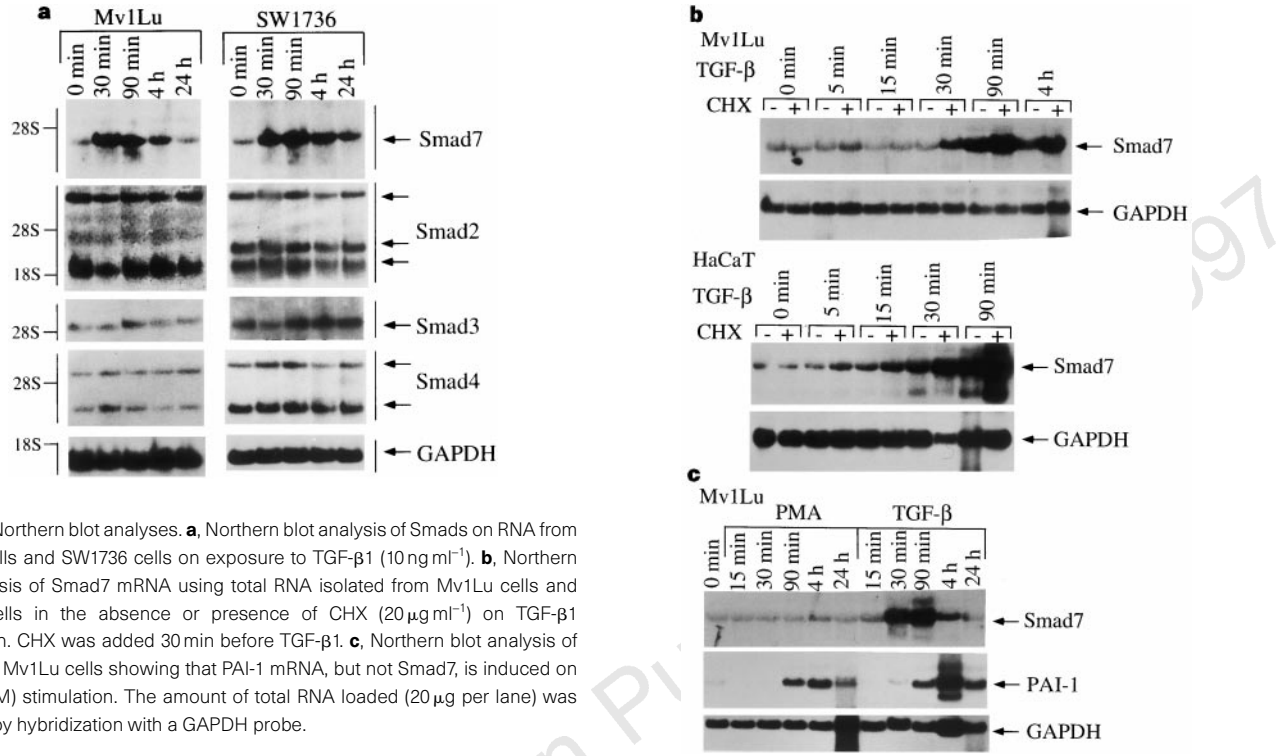


Figure 5 Northern blot analyses. **a**, Northern blot analysis of Smads on RNA from Mv1Lu cells and SW1736 cells on exposure to TGF-β1 (10 ng ml^{-1}). **b**, Northern blot analysis of Smad7 mRNA using total RNA isolated from Mv1Lu cells and HaCaT cells in the absence or presence of CHX (20 μg ml^{-1}) on TGF-β1 stimulation. CHX was added 30 min before TGF-β1. **c**, Northern blot analysis of RNA from Mv1Lu cells showing that PAI-1 mRNA, but not Smad7, is induced on TPA (10 nM) stimulation. The amount of total RNA loaded (20 μg per lane) was checked by hybridization with a GAPDH probe.

Isolation of total RNA and northern blot analysis were performed essentially as described[24].

Expression plasmids and antisera. Expression constructs for TβR-II, TβR-I, TβR-II, ActR-IB, Smad1, Smad2 and Smad3, and antisera recognizing Smads or receptors, have been described[5]. F-Smad7 was made by PCR and subcloning into pcDNA3-Flag.

Cell assays. Transfection of COS cells, metabolic labelling, immunoprecipitaiton, affinity crosslinking, [^{32}P]orthophosphate labelling of cells, and SDS–PAGE were performed as described[5].

Transcriptional response assay. Mv1Lu and R mutant cells were transfected with p3TPLux as described[5]. HaCaT cells were transiently transfected with p21 Lux[25] using Transfectam reagent from Promega (Madison, WI). In each experiment, total equal amounts of DNA were transfected. Luciferase activity was measured as described[5], and normalized for transfection efficiency. Results shown are representative of at least four independent experiments.

***Xenopus* embryo culture and manipulation.** *Xenopus* eggs were obtained and embryos microinjected and cultured as described[26]. For animal-cap assays, 200 pg of RNA encoding activin-βB or a BMP–Vg1 chimaera[27] was injected either alone or together with 400 pg of Smad7 RNA. Immunostaining of whole embryos (12/101 antibody was obtained from the Developmental Studies Hybridoma Bank under contract N01-HD-2-3144 from the NICHD; Tor70 was obtained from R. Harland), whole-mount *in situ* hybridization, RT–PCR analysis of RNA extracted from cultured animal caps, and quantification of *brachyury* expression relative to that of *EF-1a* were performed as described[6,28].

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